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Temporal and spatial control of the adenovirus proteinase by both a peptide and the viral DNA

Walter F. Mangel, Diana L. Toledo, Jianzhong Ding, Robert M. Sweet and William J. McGrath

The adenovirus proteinase (AVP) uses both an 11-amino acid peptide (pVIc)[1,2] and the viral DNA[1] as cofactors to increase its catalytic rate constant 6000-fold. The crystal structure[3] of an AVP–pVIc complex at 2.6-Å resolution reveals a new protein fold of an enzyme that is the first member of a new class of cysteine proteinases, which arose via convergent evolution.

THE ADENOVIRUS PROTEINASE has presented numerous conundrums. For example, late in adenovirus infection, virions assemble, partly from precursor proteins[4], and only upon activation of the virus-coded proteinase and cleavage of these precursor proteins does the particle become infectious. What prevents the proteinase from being active after its synthesis and before virion assembly, and how is the proteinase activated after virion assembly? There are only 50 proteinase molecules per virion[5], and between them they must cleave 2500 peptide bonds to render a particle infectious. Either enzyme, substrate or both must move; yet, the milieu within a virus particle is thought to be semi-crystalline. While possible answers at the biochemical level are presented to each of these questions, the focus of research has now shifted to answering these same questions at the structural level.

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Biochemical properties of the proteinase

Identification, cloning and expression of the gene. The gene for the adenovirus proteinase has been identified, cloned and expressed in *Escherichia coli*. Weber and coworkers isolated a temperature-sensitive mutant (H2ts-1) of adenovirus serotype 2 (Ad2), which, when grown at the nonpermissive temperature, contains precursor proteins instead of mature components[6], because the mutant proteinase is not packaged into the virion[7]. The mutation was mapped to the gene encoding the 23-kDa L3 protein[6,8], which was cloned and expressed in *E. coli*[9,10] and the resultant 204-amino acid protein purified[2,10–12].

Easy assay. A specific, sensitive and quantitative assay for the adenovirus proteinase (AVP), using rhodamine 110 as the reporting group[13], facilitated characterization of some of the biochemical properties of the enzyme (Fig. 1a). Rhodamine 110 is detectable at low concentrations, because it has a molar absorbance coefficient greater than 70 000 M^{-1} cm^{-1} and a quantum yield greater than 90%. The assay is based on the observation that AVP will cleave small peptides that contain sequences corresponding to cleavage sites on the N-terminal side of virion precursor proteins[14,15]. One consensus cleavage sequence is (L/I/M)xGG, where x is any amino acid residue and where

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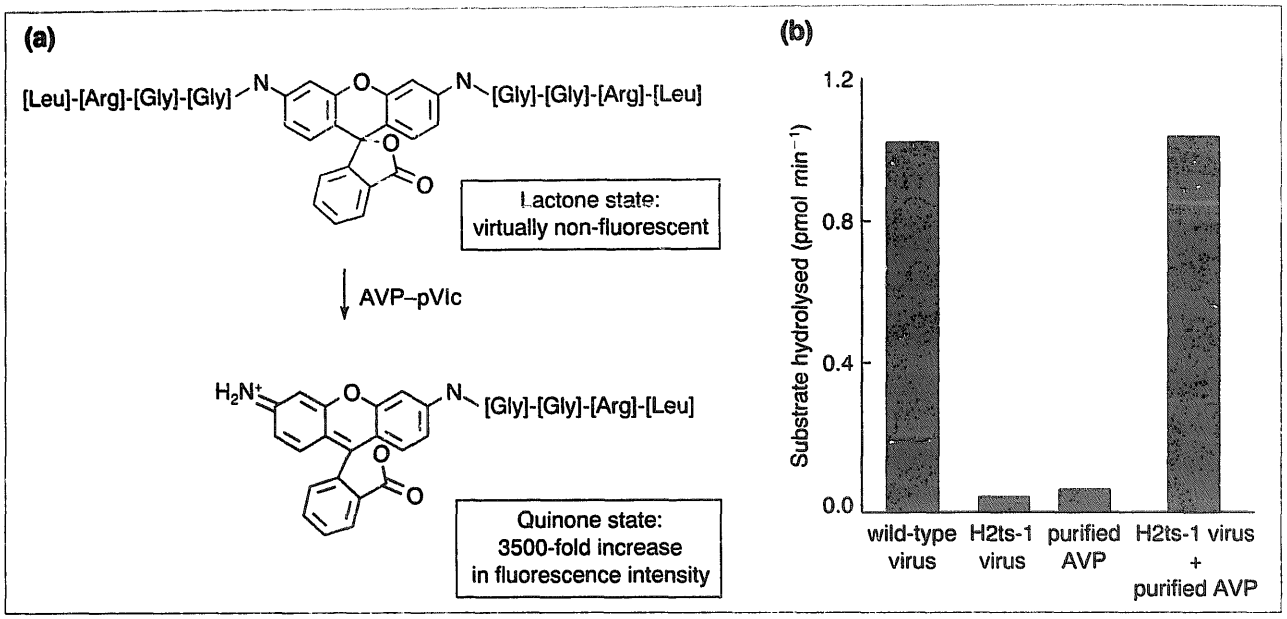

Figure 1

(a) Structure of the fluorogenic substrate (Cbz-Met-Arg-Gly-Gly-NH)$_2$–rhodamine (Cbz: benzyloxycarbonyl) and its highly fluorescent hydrolysis product Cbz-Met-Arg-Gly-Gly-NH–rhodamine[1,13,16]. The rhodamine moiety in the substrate is depicted in the lactone state. Cleavage of one of the two Gly-NH–rhodamine amide bonds in the substrate by the adenovirus proteinase (AVP) bound to an 11-amino acid peptide (pVIc) yields a hydrolysis product in which the rhodamine moiety is in the quinone state and the fluorescence intensity has increased 3500-fold. **(b)** Discovery of cofactors[1]. Equal amounts of wild-type and H2ts-1 virus were disrupted and assayed for proteinase activity. Purified, recombinant AVP (4.4 nM) had no activity. Mixing of inactive H2ts-1 with inactive AVP resulted in as much enzyme activity as in 10^{10} disrupted wild-type virus. Similar conclusions on the existence of cofactors were reached by Kemp and co-workers[2].

the bond after the second Gly residue is cleaved. The fluorogenic substrate (Cbz-Met-Arg-Gly-Gly-NH)$_2$–rhodamine is virtually non-fluorescent, because the rhodamine moiety is in the lactone state[1,16] (Fig. 1a). (Cbz is benzyloxycarbonyl.) Upon cleavage by AVP to Cbz-Met-Arg-Gly-Gly-NH–rhodamine, the rhodamine moiety converts to the quinone state and its fluorescence intensity increases 3500-fold. This substrate was used in assays to characterize proteinase activity in disrupted wild-type virus[1,17]. Predictably, there was significant enzyme activity in wild-type virus and no activity in H2ts-1 virus. Surprisingly, little substrate hydrolysis was observed with purified recombinant endoproteinase (AVP).

What is missing? Eventually cofactors were discovered. When assayed separately, AVP and disrupted H2ts-1 virus have little or no enzyme activity but, on mixing them together, significant enzyme activity is observed (Fig. 1b; Refs 1, 12). Therefore, there are cofactors in the virus particle required by AVP for activity. Kemp and colleagues reached a similar conclusion after observing that although purified AVP cleaves pVII to VII in the presence of Ad2ts1-infected cell extracts, no cleavage of the peptide substrate MSGGAFSW is detected with AVP alone[2]. One cofactor is the viral DNA[1]. If disrupted wild-type virus is treated with DNase and then assayed, proteinase activity is lost, but can be restored upon addition of Ad2 DNA. A second cofactor is a plasmin-sensitive virion protein, which turned out to be the 11-amino acid peptide, pVIc, from the C-terminus of the precursor to virion protein VI (Refs 1, 2). Its sequence is GVQSLKRRRCF. Kemp and co-workers used synthetic peptides to determine whether the cysteine in pVIc is essential[2] and whether a disulfide-linked dimer of pVIc is required for activity. They propose that activation of the proteinase by pVIc involves thiol–disulfide interchange. Recent evidence from Weber and colleagues[18] indicates that thiol–disulfide interchange is not absolutely required for enzyme activation, because the peptide VEGGS can activate the enzyme as well as can pVIc.

Sequence specificity of cofactors. To determine whether proteinase activity is dependent on specific nucleotide sequences, various polymers were substituted for Ad2 DNA[1]. Not only does T7 DNA substitute for Ad2 DNA, but also single-stranded DNAs, circular single- and double-stranded DNAs, and even polyglutamic acid. Neither polylysine nor the corresponding monomers of anionic polymers, such as AMP or glutamic acid, substitute for Ad2 DNA. Rather, it appears as if the requirement is for a polymer with high negative-charge density, e.g. the viral DNA in the virus particle. As for the other cofactor, peptides similar in character to pVIc do not substitute for it[1,2]. The cysteine residue in pVIc, Cys10', can form a disulfide bond with the proteinase *in vitro*[3].

DNA is a controversial cofactor. That DNA functions as a cofactor for proteinase activity[1] is disputed[2,19]. We showed that the viral DNA is a cofactor in the Ad2 virion, because proteinase activity is lost upon treatment with DNase and restored upon addition of Ad2 DNA[1]. Second, we showed processing of virion precursor proteins in disrupted H2ts-1 virus upon incubation with AVP; however, no processing occurred if the disrupted virions were pretreated with DNase before the addition of AVP[12]. The only condition in which we do not see stimulation of AVP activity by DNA is when we assay an AVP preparation that already contains polyanions.

Kinetic effects of cofactors. The cofactors affect the macroscopic kinetic constants of the interaction of AVP with the rhodamine-based fluorogenic substrates[12]. AVP alone has a small amount of activity (Fig. 2). By incubating Ad2 DNA with AVP, the Michaelis constant (K_m) increases twofold and the catalytic rate

constant (k_{cat}) threefold. By incubating pVIc with AVP, K_m increases twofold and k_{cat} increases 350-fold. With all three components together, AVP plus Ad2 DNA plus pVIc, K_m increases twofold and k_{cat} increases 6000-fold relative to those with AVP alone. Thus, the cofactors increase proteinase activity by increasing the k_{cat}, not decreasing the K_m.

Structural properties of the proteinase

Crystal structure of an AVP–pVIc complex. To determine the structural basis for the stimulation of proteinase activity by one of its cofactors, an AVP–pVIc complex was crystallized[20] and its three-dimensional structure solved at 2.6-Å resolution by X-ray crystallographic analysis using single isomorphous replacement supplemented with anomalous scattering[3]. The AVP–pVIc complex is ovoid, with three α-helices forming the wide end (Fig. 3). The narrow end contains another α-helix, and the region between comprises one central and two peripheral α-helices that interact with a β-sheet. The β-sheet consists of five β-strands from AVP; a sixth β-strand originates from the last eight amino acid residues of pVIc. There is extensive contact between AVP and pVIc – 34 hydrogen bonds, four ion pairs and a disulfide bond between Cys104 of AVP and Cys10′ of pVIc. The AVP–pVIc complex appears to consist of two domains; pVIc forms a 'strap' that helps to position the two domains. The molecular surface of the AVP–pVIc complex has four large clusters of positive-charge density ranging in area from 45 to 65 Å^2: these are potential DNA-binding sites. The shortest distance between clusters (~24 Å) is commensurate with the rise of a single turn of double-stranded, helical DNA.

Class of proteinase? AVP appears to represent a new type of proteinase. The sequence of the gene for the proteinase[21,22] is not related to any gene sequences in the databases. Inhibitor profiles of enzyme activity give ambiguous results. Comparing the structure of AVP–pVIc with all unique protein molecules in the Brookhaven Protein Data Bank revealed no equivalent structure, suggesting that AVP represents a new family of protein molecules. For example, the fold of AVP–pVIc looks different from the fold of papain (Fig. 4a,b). However, we noticed a helix and several β-strands within the central region of AVP–pVIc that appear to be in similar positions in papain (Fig. 4c,d). These include a long, central α-helix flanked by several anti-parallel β-strands. When the common secondary structures are aligned (Fig. 4e), and the

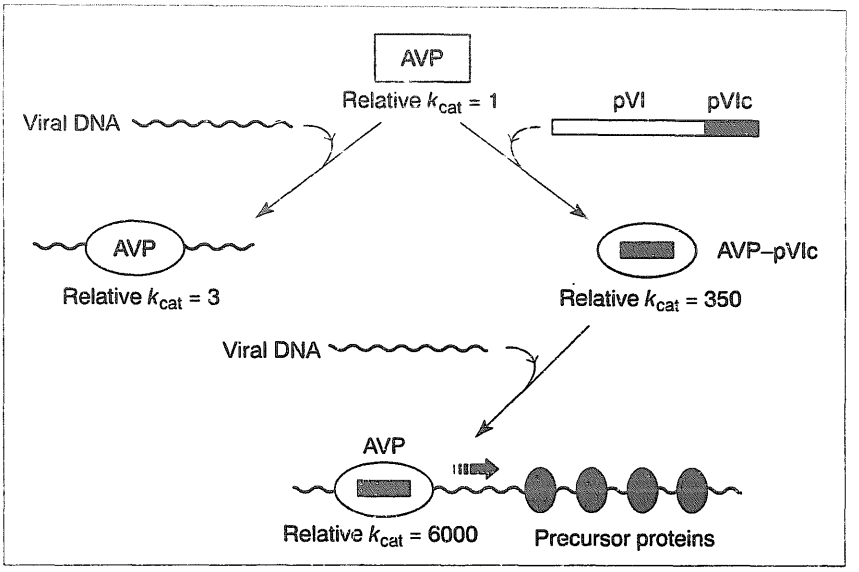

Figure 2

Model for the temporal and spatial control of enzyme activity by cofactors[1]. The adenovirus proteinase (AVP) has a low k_{cat}, 0.055 × 10^{-3} s^{-1} (Ref. 12), which increases threefold upon binding to the viral DNA. This increase in activity is sufficient for the enzyme to cleave pVI bound to the viral DNA, liberating the 11-amino acid peptide cofactor pVIc. The peptide then binds to AVP and increases the k_{cat} 350-fold. The AVP–pVIc complex binds to the viral DNA, and the k_{cat} increases 6000-fold. This active ternary complex then moves along the DNA via one-dimensional diffusion, cleaving virion precursor proteins.

Figure 3

The secondary structure of the adenovirus proteinase (AVP)–pVIc complex[3]. α-Helices are labeled H1 through H7 and colored blue; β-strands are labeled S1 through S7 and colored yellow. The termini of AVP are denoted as N-term and C-term, those of pVIc as n-term and c-term. The pVIc peptide is colored magenta. Sidechains are shown only for the active-site residues Cys122, His54 and Glu71. The package used to produce this figure was MOLSCRIPT[46].

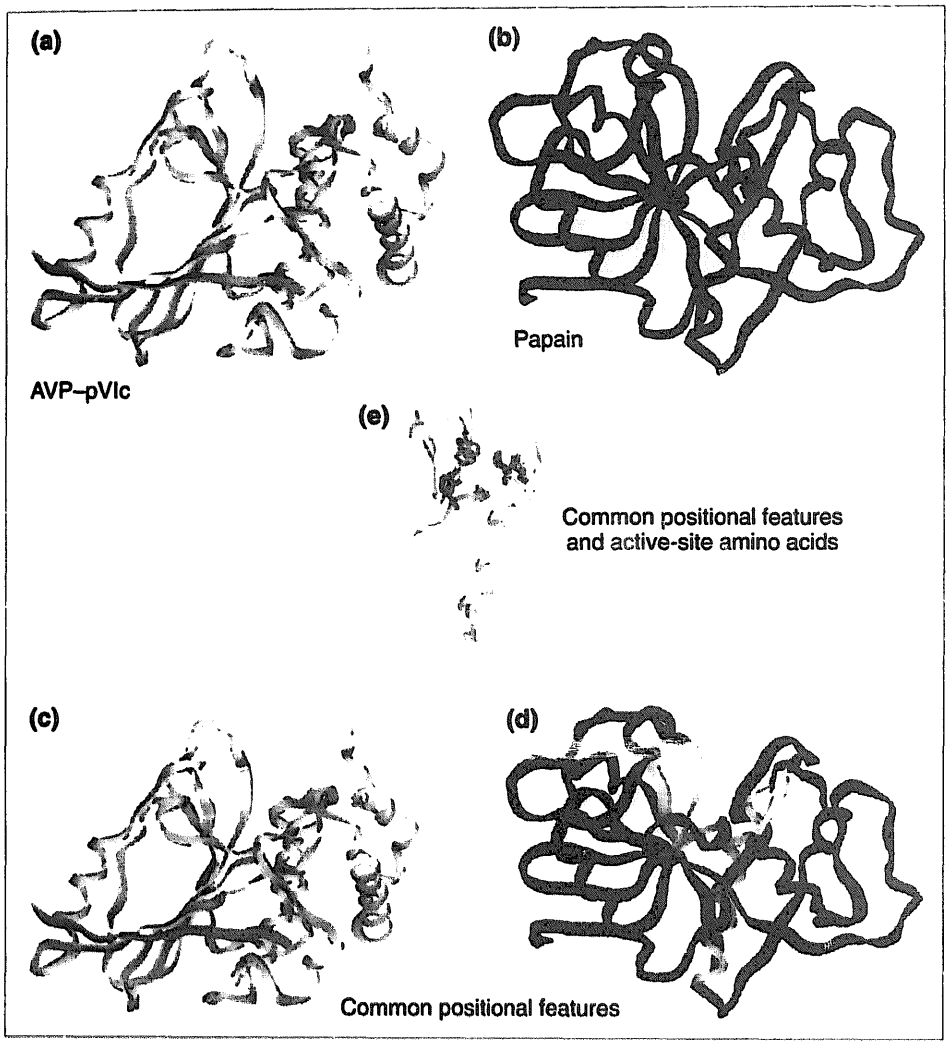

Figure 4

Convergent evolution leading to a new class of cysteine proteinases[3]. The Cα backbone atoms of the adenovirus proteinase (AVP) with pVIc in orange **(a)** and papain **(b)** indicate the two proteins have different folds. However, within them are common positional features, highlighted by the green in AVP **(c)** and the pink in papain **(d)**. In **(e)**, the active-site residues involved in catalysis for both AVP and papain are shown originating from the common positional features. This figure was produced using SETOR[47].

amino acids of the active-site region of papain and those in the same position in AVP are compared, it is clear what the type of proteinase and the nature of the active site of AVP is.

Locating the active site. Cys122 of AVP is in an identical position to the nucleophilic Cys25 of papain (Fig. 5). Furthermore, two other residues of AVP (His54 and Glu71) are in identical positions to those of His159 and Asn175, two other residues of papain involved in catalysis[23]. Even Gln19 of papain, presumed to participate in the formation of an oxyanion hole[24], aligns with Gln115 of AVP. The main-chain nitrogen atoms of the two active-site Cys residues also match; in papain this atom is proposed to join with Gln19 to form the oxyanion hole[25]. This remarkable juxtaposition of catalytic elements strongly suggests that AVP employs the same catalytic mechanism as papain[26], and that AVP is the first member of a new class of cysteine proteinases and an example of convergent evolution.

There are other indications that AVP is a cysteine proteinase: (1) The putative active site is on the surface of the molecule (Fig. 6). It lies within a 25-Å long, bent groove that is ~8 Å wide; Cys122 and His54 lie in the middle of that groove. (2) These two amino acids are conserved among adenovirus serotypes. (3) A 3.5-Å hydrogen bond is formed between atoms S of Cys122 and Nδ of His54. (4) These two amino acids probably form a Cys–His ion pair, like the nucleophilic Cys–His ion pair in papain[27], because a thiolate anion in AVP can be titrated at pH5.0 with dithiodipyridine[12]. (5) Replacement of Cys122 by an Ala residue results in loss of enzyme activity[28]; this indicates that Cys122 is required for enzyme activity. Cys104 is not the active-site nucleophile[4,29], as it clearly forms a disulfide bond with pVIc[3]. (6) Glu71, probably the third member of the triad involved in catalysis[23], lies on the other side of the imidazole ring of His54 from Cys122. A 2.7-Å hydrogen bond is formed between atoms Oε of Glu71 and Nε of His54. Glu71 is replaced only by Asp among adenovirus strains.

New protein fold, new class of proteinase. The structure of the adenovirus proteinase is unique. Despite the similarities with papain in the positions of the amino acid residues involved in catalysis, the sequential order of these amino acid residues in the polypeptide chain is different. In AVP, the triad involved in catalysis is His54, Glu71 and Cys122, whereas in papain the order is Cys25, His159 and Asn175. Thus, AVP is a cysteine proteinase in a different class from both the papain superfamily and the viral cysteine proteinases: it belongs to the subclass His/Cys[30]. Until recently, there were four known groups of enzymes with active sites containing catalytic triads generated by convergent evolution: the eukaryotic serine proteinases, the cysteine proteinases, subtilisins and the α/β hydrolase-fold enzymes[31]. Because of its unique fold, AVP must represent a fifth group.

Action at a distance. Surprisingly, pVIc, which exerts powerful control on the rate of catalysis, binds quite far from the active-site residues involved in catalysis; the pVIc cysteine residue, which forms a disulfide bond with Cys104 of the main chain, is 32 Å away from the active-site nucleophile Cys122 (Fig. 6). The residue of pVIc closest to the active site is Val2′, whose sidechain is 14.5 Å away from Cys122. Upon the binding of pVIc, what far-reaching changes occur in the active site that lead to a large increase in the catalytic rate constant for substrate hydrolysis?

Other roles of the proteinase

Aside from processing virion precursor proteins late in infection, AVP might

have other functions, e.g. the proteinase has been shown to cleave actin[11] and cytokeratins[32], implying a role in cell lysis. The cleavage of actin by AVP in the absence of pVIc[11] was interpreted as indicating the enzyme does not absolutely need a peptide cofactor to cleave a protein substrate. There is also evidence that AVP is involved early in infection, such as in virus entry[33] and in disassembly of an incoming virus particle[34].

Role of proteinase in virus maturation

Model for late functions of cofactors. Functions of the cofactors might be to regulate the temporal and spatial activity of the enzyme (Fig. 2). Our working hypothesis is that the enzyme is initially synthesized with negligible activity. Presumably, if it were active before virion assembly, it would cleave virion precursor proteins, thereby preventing virion assembly. Late in infection, virion proteins, including precursor proteins, assemble into 'empty capsids'[35]. Then, the core proteins and AVP bound to the viral DNA are encapsidated, generating 'young virions'. Binding to DNA increases the k_{cat} of AVP threefold. It is in these 'top components' that the proteinase is activated and the precursor proteins are processed to yield mature, infectious virus. This could occur by pVI binding to the viral DNA[36] such that AVP already bound to the viral DNA can excise pVIc (Fig. 2). The released pVIc could then bind AVP. The AVP–pVIc complex next binds to the viral DNA, and this ternary complex, AVP– pVIc–DNA, is a fully active proteinase.

A proteinase that acts like RNA polymerase. How can 50 fully-activated proteinases[5], bound to the viral DNA inside the virion, cleave 2500 peptide bonds in precursor proteins to render a virus particle infectious? Perhaps, the viral DNA serves as a guidewire, next to which are the 2500 processing sites that must be cleaved. The proteinase complex could then slide along the viral DNA cleaving the precursor proteins (Fig. 2). The binding of AVP–pVIc complexes to DNA is not sequence specific[1], a property that allows the proteinase to move along the viral DNA. This would be analogous to the binding of the *E. coli* RNA polymerase holoenzyme to non-promoter DNA sequences[37]. RNA polymerase binds to non-promoter DNA sequences with a K_d of 100 nM, and the polymerase slides along the DNA via one-dimensional diffusion[38] until it locates a promoter. In the case of AVP, it slides along the viral DNA encountering precursor cleavage sites.

Outlook

Four years ago, there was no precedent for the regulation of proteinase activity by a small peptide and by DNA, but the cofactor story of AVP is now 'less unique'. For example, the NS3 protein of hepatitis C virus, a positive-sense RNA virus, is a serine proteinase whose activity is enhanced by a cofactor, the 54-amino acid residue NS4A protein. A 12-residue synthetic peptide, comprising amino acids 12–33 of NS4A, forms a complex with the NS3 proteinase domain and activates the enzyme so that it can cleave at certain processing sites[39]. Whether virus-coded proteinases other than AVP use nucleic acids to stimulate and to localize proteinase activity is being ascertained, and the three-dimensional structures of many virus-coded proteinases have recently been published[40–45]. Therefore,

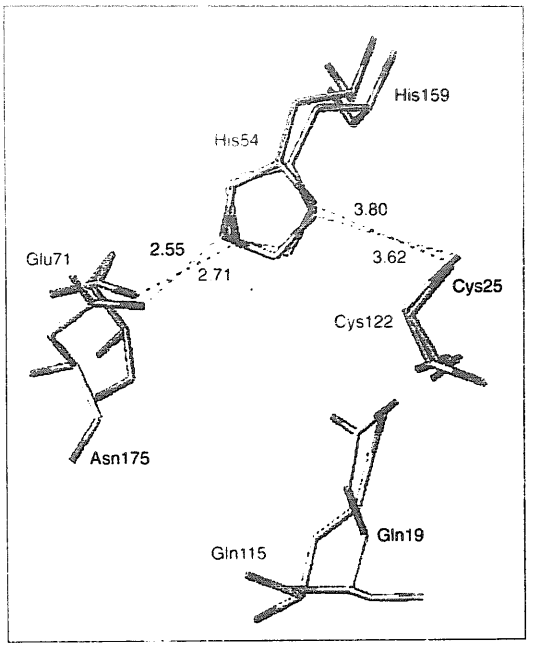

Figure 5
Juxtaposition of the active-site residues of the adenovirus proteinase (AVP) and papain[3]. The residues involved in catalysis in papain are shown after alignment of the papain molecule to fit the equivalent residues in AVP. Active-site residues and bond distances (in Å) are in white for papain and in magenta for AVP. The package used to produce this figure was O (Ref. 48).

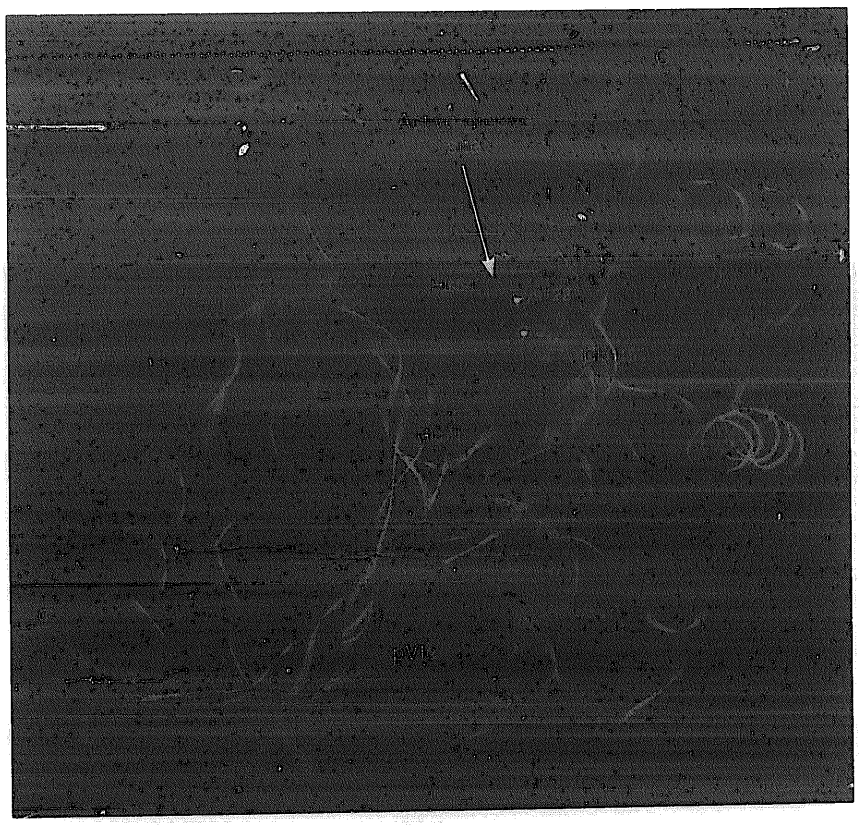

Figure 6
Crystal structure of the adenovirus proteinase (AVP) with its 11-amino-acid cofactor pVIc[3]. The fold of AVP is shown in orange, pVIc in green. In the active-site groove are the four amino acid residues involved in catalysis. The N-terminus (N) and the C-terminus (C) of AVP are located in the upper-right side of the figure. The package used to produce this figure was SETOR[47].

elucidating the regulation of enzyme activity at the structural level is imminent. And, of course, all this information is being used for structure-based rational drug design.

Acknowledgements

This article is dedicated to the memory of Gregorio Weber. The research was supported by National Institute of Allergy and Infectious Diseases Grant AI41599, the Office of Health and Environmental Research of the United States Department of Energy, the National Science Foundation, and the Department of Energy's Office of Science Education and Technical Information.

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